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OBPC SYMPOSIUM: MAIZE 2004 & BEYOND – PLANT VIRUS-BASED VECTORS IN AGRICULTURE AND BIOTECHNOLOGY

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Summary

The study of plant viruses and their interaction with the plant host has contributed greatly to our understanding of plant biology. The recent development of plant viruses as transient expression vectors has not only enhanced our understanding of virus biology and antiviral defense mechanisms in plants, but has also led to the use of plant viral-based vectors as tools for gene discovery and production of recombinant proteins in plants for control of human and animal diseases. An overview of the state-of-the-art of viral expression systems is presented, as well as examples from our laboratory on their use in identifying nuclear targeting motifs on viroid molecules and development of therapeutic proteins for control of animal diseases.

Key words: pharming; plant-derived biologics; plant virology; recombinant vaccines; VIGS.

Introduction

The conventional approach for expression of foreign genes in plants involves the stable integration of specific genes by transformation and regeneration of plant tissues using one of several techniques. Unfortunately, this approach is not applicable to all species at this time. Transient expression using episomal plant virus-based vectors has been developed as an alternative for expression of foreign genes and it offers the advantages of speed and versatility and a high level of gene expression.

The small genome size of most plant viruses, in the range of 1–18 kb, implies highly efficient coding capacities of their genomes. Efficient replication, genome expression, and movement of viral genomes are achieved by the use of multifunctional control sequences. These sequences include transcriptional promoters, translational enhancers, proteins and nucleic acid motifs required for replication, translation, proteolytic processing, and intracellular, cell-to-cell, and long-distance movement (Mushegian and Shepherd, 1995). These elements have provided valuable tools for plant molecular biology, e.g., the *Cauliflower mosaic virus* (CaMV) 35S transcriptional promoter (Odell et al., 1985) and translational enhancer from *Alfalfa mosaic virus* (AlMV) (Jobling and Gehrke, 1987).

The ability to molecularly clone and manipulate plant viral genomes as full-length, infectious cDNAs has led to significant increases in our understanding of the expression of viral genomes and the interaction of a virus with its host for replication and movement functions, induction of disease, and resistance responses. The potential of viruses as autonomously replicating vectors for the delivery and expression of foreign genes *in vivo* has also been exploited (Scholthof et al., 1996; Pogue et al., 1998, 2002).

The earliest plant viral vectors were derived from CaMV, an isometric virus with a double-stranded DNA genome that replicates through an RNA intermediate (Gronenborn et al., 1981; Brisson et al., 1984). Single-stranded DNA geminiviruses have also been explored as plant viral-based vectors. These DNA-based vectors suffer from a limited host range, rapid deletion of foreign sequences in some cases, and size constraints of the inserts they are able to accommodate (Hayes et al., 1989; Scholthof et al., 1996). Despite these limitations, Palmer et al. (1999) demonstrated that a geminivirus-based replicon based on gene replacement replicated efficiently in biolistically transformed cells and could be useful for long-term gene amplification in cereal cell cultures.

Nanoviruses are ssDNA plant viruses whose major hosts are legumes or *Musa* species (Vetten et al., 2001). The genome consists of multiple circular DNAs of 1000 nucleotides each, the individual DNA molecules carry a single gene (Gronenborn et al., 2002), and the viruses replicate in the nucleus via a double-stranded DNA intermediate. Aronson et al. (2002) constructed a series of episomal vectors based on the nanovirus *Faba bean necrotic yellows virus* to demonstrate *in planta* interactions between two proteins, one of which was a plant protein in the ubiquitin-dependent protein turnover pathway. As the natural host range of this virus includes *Arabidopsis thaliana*, this system could be very useful in gene discovery experiments.

Most plant viruses are composed of RNA genomes, and these have been used with greater success, despite early doubts about their usefulness and stability because of the low fidelity of RNA polymerase (van Vloten-Doting et al., 1985). In fact, the mutational

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frequency in a foreign gene sequence expressed from a *Tobacco mosaic virus* (TMV)-based vector was found to be relatively low (Kearney et al., 1993). However, the stability of foreign gene sequences depends upon the virus used to develop the virus-based vector, the nature of the sequence, and the placement of the sequence. Examples will be discussed later. As the majority of plant viruses consist of single-stranded, positive-sense RNA genomes, manipulation requires production of an infectious cDNA clone.

Vector strategies

Plant RNA virus-based vectors that employ different genome expression strategies have been constructed and the advantages and disadvantages of these expression systems have become apparent following their use (Scholthof et al., 1996). Several strategies have been used for expression of model proteins, sense and antisense RNAs, vaccine antigens, or therapeutic proteins from plant viral vectors.

Gene replacement vectors, in which a 'non-essential' viral gene is replaced by a foreign gene, have been developed using several plant viruses (Scholthof et al., 1996; Pogue et al., 2002). For example, the TMV coat protein (CP) was replaced with the bacterial chloramphenicol acetyl transferase gene (Takamatsu et al., 1987), however, the virus was debilitated for systemic movement. While useful in a protoplast system for production of biomedicals, this vector is not of practical use in the field.

The most widely used strategy for expression of a foreign gene is where the gene is driven by a separate subgenomic RNA promoter. This strategy takes advantage of the natural expression of the CP from a subgenomic RNA which is synthesized from the genomic RNA during virus infection. Duplication of the subgenomic RNA promoter followed by fusion of the foreign gene to one of the promoters results in expression of both the coat protein and foreign protein on separate, non-encapsidated mRNAs. Although early TMV-based vectors containing a homologous duplicated CP subgenomic promoter rapidly lost the foreign insert (Dawson et al., 1989), improvements in vector design by substitution of the homologous promoter with a subgenomic promoter from a related virus have improved stability of inserted genes (Donson et al., 1991; Shivprasad et al., 1999; Rabindran and Dawson, 2001). *Potato virus X* (PVX) has also been used extensively as a viral-based vector using this strategy, both for production of foreign proteins (Franconi et al., 2002), as well as for gene discovery and silencing, as discussed below.

Translational fusions of the gene of interest with a viral structural gene have been utilized where a partial or complete open reading frame (ORF) is fused to either the N- or C-terminus of a viral structural gene (peptide presentation), either with or without a proteolytic cleavage site allowing processing of the fused sequence (e.g. Gopinath et al., 2000). Alternatively, the additional sequence is expressed as a read-through fusion, so that both WT and modified viral proteins are produced (Hamamoto et al., 1993). A strategy that has been quite successful is the addition or substitution of a short antigenic region (an epitope) of the protein of interest at either the N or C terminus of the viral CP, or in an internal loop that is exposed on the virion surface (epitope display). This strategy has been used with TMV, PVX, and *Cowpea mosaic virus* (CPMV), among others (Johnson et al., 1997; Porta and Lomonossoff, 1998; Pogue et al., 2002).

With multi-component plant viruses and viruses containing a defective RNA (dRNA), functional complementation can be used so that a genome segment expressing the gene of interest also complements a defective viral component in such a way as to minimize the probability of recombination to recreate a functional WT virus. An example is our use of *Cucumber mosaic virus* (CMV) with two copies of RNA 3, RNA 3A with green fluorescent protein (GFP) in place of the ORF 3a movement protein (MP) but with a functional CP, and RNA 3B with a functional 3a MP and the gene of interest in place of the CP gene (Zhao et al., 2000). Functional complementation of a dRNA or DNA by a wild-type virus was shown by Raffo and Dawson (1991), who demonstrated that WT TMV could support the systemic replication of defective TMV amplicons, without noticeably interfering with WT symptoms or replication.

Trans-complementation of a defective virus through transgene expression in the host plant was first demonstrated with AlMV where transgenic plants expressing AlMV RNAs 1 and 2, which encode the replicative functions, were able to support a productive infection if inoculated with RNA 3 (Bol, 1999; Sanchez-Navarro et al., 2001). This system has the added advantage of biological containment, in that virus particles produced in this system are not infectious to non-transgenic plants as the transgenic RNAs 1 and 2 lack the terminal sequences required for infectivity.

Chimeric viruses can also be constructed, where the CP of one virus is expressed as a fusion protein for peptide presentation or epitope display in addition to the homologous WT CP required for efficient long-distance movement. Yusibov et al. (1997) employed this strategy to produce candidate HIV and rabies virus vaccines. Antigenic determinants from rabies virus and HIV-1 were engineered as fusions with the AlMV CP and expressed from a TMV vector. The resulting peptide-modified AlMV CP assembled into virus-like particles (VLPs) presenting the rabies or HIV peptides in a regular array on the VLP surface.

Viral amplicons can also be incorporated into the plant genome by transformation or via Agro-infiltration. Liu and Lomonossoff (2002) delivered two subgenomic components of CPMV from a mixture of separate *Agrobacterium* suspensions. Multicomponent isometric plant viruses such as CPMV suffer from packaging constraints on the size of potential inserts, but the addition of GFP to RNA 2 of CPMV (Liu and Lomonossoff, 2002) resulted in an RNA 2 that is still shorter than the independently packaged RNA 1, so this system appears to offer some flexibility. Initial failure of the transgenic viral amplicon approach as a result of RNA silencing (with protein expression similar to a standard transgene; Angell and Baulcombe, 1997) was reversed by inclusion of potyviral HC-Pro (a viral suppressor of RNA silencing) in the 'amplicon-plus' system, with expression of a reporter gene to about 3% of total protein (Mallory et al., 2002).

Finally, Marillonnet et al. (2004) and Gleba et al. (2004) describe expression systems in which viral functions not needed for expression are removed, and functional complementation is used to mix and match functions from different sources. Expression can be optimized, and biocontainment built into the system to minimize or prevent infectivity to other plants. An efficient means of delivery of multiple components by Agro-infiltration, combined with *in planta* recombination, was shown to confer high yields, and to allow great flexibility in comparing variants in one or more of the system components. Incorporation of suitable constructs as transgenes allows induction of replication and expression as required at

particular developmental stages or by application of an inducer (Gleba et al., 2004).

Examples of Uses in Biotechnology and Agriculture

Functional analysis of heterologous sequences and gene discovery. There are many examples in the literature of the employment of viral-based vectors to examine the function of plant genes (reviewed in Pogue et al., 2002). For example, Hong et al. (1997) describe a novel strategy for controlling expression of a transgene encoding a plant ribosomal inactivating protein using a virus-inducible geminivirus promoter and transactivation of that promoter using a transactivator expressed from a PVX vector. This strategy is particularly useful for the regulation of metabolites which could be toxic to the plant or that may inhibit growth and development of the plant.

The production of novel compounds was demonstrated by Kumagai et al. (1998) by overexpression of the cDNA encoding the pepper capsanthin-capsorubin synthase in *N. benthamiana* using PVX. The enzyme was able to use endogenous carotenoids for production of an orange-red phenotype and expression of novel carotenoids capsanthin and capsorubin, indicating the potential for remodeling photosynthetic membranes for better photoreactivity.

Using another approach, Fridborg et al. (2004) screened 12 000 enhancer trap *A. thaliana* plants for genes up-regulated by virus infection. *Tobacco rattle virus* (TRV) tagged with GFP was inoculated onto plants carrying T-DNA insertions composed of a minimal 35S CaMV promoter and a GUS (β-glucuronidase) reporter gene. After rub inoculation with the TRV construct, one line was found to contain a pathogen-inducible gene containing a pathogen-responsive element implicated in defense responses.

Utilizing the cytoplasmic PVX vector and a GFP reporter, we have demonstrated the nuclear targeting of heterologous RNA sequences by *Potato spindle tuber viroid* (PSTVd), a single-stranded, covalently-closed circular RNA molecule of 359 nt that replicates and accumulates in the nucleus of infected cells (Zhao et al., 2001). The nuclear targeting signals or motifs on PSTVd can be harnessed for biotechnology applications and for the identification of host factors that function in nuclear import of RNA molecules.

Virus-induced gene silencing. Virus-induced gene silencing (VIGS) is a technology that takes advantage of an RNA-mediated antiviral defense mechanism in plants. It is increasingly being used to generate transient loss-of-function assays to analyze gene function and for high-throughput functional genomics and gene discovery (Angell and Baulcombe, 1997; Thomas et al., 2001; Lu et al., 2003; Brigneti et al., 2004; Faivre-Rampant et al., 2004; Liu et al., 2004). Kumagai et al. (1995) first demonstrated that antisense transcripts made in the cytoplasm of a cell can down-regulate endogenous gene expression. It has been successfully applied to *Arabidopsis* (Lu et al., 2003) and the *Solanaceae* family, including tobacco (Baulcombe, 1999), tomato (Liu et al., 2002), and potato (Brigneti et al., 2004; Faivre-Rampant et al., 2004), using different viral-based vectors.

Thomas et al. (2001) examined the size limit of sequence identity required for post-transcriptional gene silencing (PTGS) between the target gene and the silencing sequence delivered using a PVX vector. Short sequences of 23 nucleotides in length and complete identity were able to initiate silencing of several genes in *Nicotiana benthamiana*, although the silencing occurred in the absence of DNA methylation thought to be correlated with PTGS. A method designed to enhance the VIGS phenotype by increasing the level of double-stranded (ds) RNA molecule production was reported by Lacomme et al. (2003). This strategy employs the incorporation of 40–60 base direct inverted repeats of the phytoene desaturase gene into a TMV-based vector and a *Barley stripe mosaic virus*-based vector. The VIGS resulted in generation of a more robust loss-of-function phenotype than expression of single-stranded RNA molecules.

VIGS was used by Liu et al. (2004) to characterize known defense genes in tobacco involved in resistance gene signaling. TRV constructs encoding protein kinases and transcription factors were used to down-regulate the expression of corresponding endogenous genes in tobacco resulting in modified plant resistance responses to TMV.

Disease resistance. PVX has been used to rapidly characterize the phenotypic result of overexpression of plant genes involved in defense responses against pathogens. Rommens et al. (1995) expressed the tomato disease resistance gene, Pto, which confers resistance to *Pseudomonas syringae* pv. tomato, and a Pto homolog, Fen. Expression of Pto from the virus genome failed to elicit resistance in tomato to the bacterial pathogen, indicating that PVX was not suitable for the study of Pto. However, expression of Fen resulted in sensitivity to the insecticide fenthion and allowed further structure/function characterization of the Fen gene in the PVX-based vector.

An antimicrobial defensin from wasabi was expressed from a PVX vector and was shown to retain functional activity against two phytopathogenic fungi *in vitro* (Saitoh et al., 2001). We have evaluated the potential of two naturally occurring antimicrobial peptides, snakin-1 and defensin PTH1, which have different reaction spectrums and sensitivities, for their ability to inhibit bacterial growth (Zhao and Hammond, 2000). The peptides were expressed as a single protein, that undergoes self-cleavage by an embedded foot and mouth disease virus 2A protease, from a PVX-based vector. Preliminary results showed that the engineered genes suppressed multiplication and establishment of *Clavibacter michiganensis* subsp. *sepdonicus* bacteria in plants.

Pathogen factors that produce disease symptoms or elicit a plant defense response can also be investigated using virus-based vectors. A necrosis-inducing protein from the oomycete fungus, *Phytophthora sojae*, was identified by transient expression of fungal expressed sequence tags (ESTs) from a binary transformation vector containing the PVX genome (Qutob et al., 2002). The PVX-binary plant transformation vector coupled with Agro-inoculation has been used as a tool in many studies, including gene silencing and co-suppression, as well as high-throughput functional screening (Takken et al., 2000).

Biomedical production for human and animal diseases and regulatory considerations. There is an increasing demand for pure, properly folded, and biologically active therapeutic proteins for medical and veterinary applications. Current production systems include growth of animal or human pathogens in cell cultures (or chicken eggs in the case of flu virus vaccine) and subsequent inactivation for use as vaccines, and synthesis of recombinant proteins in cell bioreactors or yeast systems. These systems are very expensive and scale-up costs are quite high. As an alternative, plants are being evaluated as recombinant protein bioreactors.

There are two mechanisms for producing recombinant proteins in plants: transgenic and transient expression using viral-based vectors, and both have been utilized for producing therapeutic proteins (Kumagai et al., 1993), monoclonal antibodies (Verch et al., 1998; McCormick et al., 1999), and vaccines (see reviews by Awram et al., 2002; Pogue et al., 2002; Walmsley and Arntzen, 2000, 2003; Streatfield and Howard, 2003).

In our laboratory, we have expressed an animal receptor protein for use in treatment of mastitis in dairy cows. Coliform mastitis is one of the most common forms of environmental mastitis in dairy cows, accounting for 40–50% of all clinical cases of mastitis (Hogan and Larry Smith, 2003). Important Gram-negative organisms in mastitis include: *Escherichia coli*, *Klebsiella pneumonia*, *Serratia marcescens*, and *Pseudomonas aeruginosa*. We have expressed a recombinant form of CD14, a high-affinity receptor for the complex of lipopolysaccharides (LPS) and LPS-binding protein in plants. The secreted form of CD14 (sCD14) binds and neutralizes LPS from *E. coli* and other coliform bacteria, and prevents development of acute endotoxin shock in cows, as well as intramammary infection by coliform organisms (Lee et al., 2003). Transient expression in *N. benthamiana* from a PVX vector yielded a biologically active recombinant protein as demonstrated *in vitro* by induction of apoptosis and interleukin-8 production in bovine endothelial cells and *in vivo* as shown by reduced clinical symptoms in mammary quarters challenged with *E. coli* (Nemchinov et al., manuscript in preparation).

The regulatory considerations for products made in bioengineered plants, using either engineered viruses or transgenic plants, are, for the most part, the same as those for other therapeutics or vaccines, except for issues that may be unique for production of the products in plants (Stein and Webber, 2001; Peterson and Arntzen, 2004). Other issues that may need to be addressed relate to the use of bioengineered feed in animals where meat or milk is destined to be used as human food.

The potential for use of plant-derived biologics in human and animal health is high. However, in spite of the potential of plants as bioreactors, one would also need to show that the plant-derived product is clearly advantageous either for efficacy, ease of delivery and/or cost. Several of the reports reviewed in this chapter provide the 'proof of principle' that the products are efficacious. However, cost is clearly a factor when replacements are considered for traditional veterinary vaccines, some of which are currently available for pennies per dose. With that said, the most likely near-term possibilities for commercialization of plant-derived vaccines and therapeutics will be veterinary products.

Potential of Viral-based Vectors for Monocots

Barley stripe mosaic hordeivirus, a tripartite, monocot-infecting virus, possesses a CP that is dispensable for long-distance movement. However, replacement of the CP with a foreign gene results in a drastic reduction in infection (Joshi et al., 1990). *Wheat streak mosaic virus*, a potyvirus, was successfully engineered into a viral-based vector for wheat, barley, oats, and maize (Choi et al., 2000). Foreign inserts were placed between the nuclear inclusion and coat protein domains of the viral polyprotein, and were flanked by protease cleavage sites. Systemic infection of viral constructs containing the NPTII gene was stable for 18–30 d post-inoculation, however, a GUS gene was unstable when expressed as a nuclear inclusion b/GUS fusion.

Other potential expression vectors for monocots could be developed from viruses for which the complete nucleotide sequence is known and where related virus family members have been used successfully as virus-based vectors. These include *foxtail mosaic potexvirus* (Bancroft et al., 1991), *maize dwarf mosaic potyvirus* (Kong and Steinbiss, 1998), and Johnson *grass mosaic potyvirus* (Gough and Shukla, 1993).

Advantages and Disadvantages of Viral-based Vectors for Gene Expression

Despite their utility, there are some problems associated with insert stability, low levels of foreign gene expression, and co-suppression gene silencing (Shivprasad et al., 1999; Vance and Vaucheret, 2001; Voinnet, 2001). For example, Shivprasad et al. (1999) showed that heterologous sequences affect foreign gene expression in TMV-based vectors. As most plant viral-based vectors replicate in cytoplasm, they cannot be used in promoter and transcriptional factor studies unless both the promoter and transcriptional factor are both delivered via the virus-based vector.

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